IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Mehta, Ashwin
Art Unit	:	1638
Applicants	:	Parrott and LaFayette
Serial No.	:	09/802,208
Docket No.	:	UGA-855R
Filed	:	03/08/2001
For	:	Arabitol or Ribitol As Positive Selectable Markers

Commissioner for Patents
Alexandria, VA 22313

DECLARATION OF PETER LAFAYETTE, PhD.

I, Peter LaFayette, Ph.D. hereby declare and say as follows:

THAT, I am employed as Assistant Research Scientist, Department of Crop and Soil Sciences at University of Georgia, Athens, Georgia;

THAT, I earned my Ph.D. in Plant Physiology in 1988, from University of California at Davis, Davis, California;

THAT, I am one of the above-named Applicants and inventors of the subject matter described and claimed in the above-identified patent application;

THAT, by virtue of my educational and employment background, my attendance at seminars, my ongoing research, my continuing review of scientific periodicals and journals, and through correspondence with professional colleagues, I am aware of the level of skill of one ordinarily skilled in the art of plant molecular biology;

THAT, I have studied the application Serial No. 09/802,208 and all office actions which have been issued during prosecution of this application (including cited

references), as well as all responses which have been filed on the Applicants' behalf, and being thus duly qualified declare as follows:

1. I was involved in the studies conducted in Dr. Wayne Parrott's laboratory at the University of Georgia:

Cloning and characterization of *E. Coli atl*D gene

E. *Coli* strain C genomic DNA was cut with *Pst*I, ligated to pBluescript cut with *Pst*I and used to transform *E. Coli* DH10B. Transformants were selected with minimal media containing arabitol as the sole carbon source. The culture was streaked on arabitol medium plates to obtain single colonies containing the arabitol operon insert. Plasmids were isolated and the inserts sequenced. The sequence of the dehydrogenase, *atl*D, was assembled after comparing sequence runs with the sequence of the arabitol operon from *K. pneumoniae* via BLAST analysis (Altschul et al., 1997).

Construction of plant transformation vectors with the *atl*D gene

A synthetic version of the gene was synthesized to optimize codon expression in plants and to remove cis-active DNA motifs. For tobacco transformation, the synthetic *atl*D (*atl*D-S) was subcloned into pUPC6 thus placing *atl*D-S under the control of the Ubi3 promoter and Ubi3 terminator. The Ubi3P-*atl*D-S-Ubi3T construct was then subcloned into pKG, a modified pCAMBIA1305.2 vector (CAMBIA, Canberra, Australia) in which the *hph* gene was replaced with *npt*II, to produce pKGA. pKGA confers the abilty to grow on arabitol by virtue of the *atl*D-S driven by the *ubi*-3 promoter and confers resistance to kanamycin by virtue of *npt*II driven by the CaMV35S promoter. For the transformation of rice, *hph* of pCAMBIA 1305.2 was replaced with *atl*D-S forming pAS1305.2.

Tobacco transformation and selection

Agrobacterium tumefaciens strain EHA105 (Hood et al., 1993) was transformed by electroporation with plasmid pKGA and subsequently used to transform *Nicotiana tabacum* cv. KY160 by leaf disc transformation (Horsch et al., 1985). Six tobacco leaf discs were co-cultivated with *Agrobacterium* on Tobacco Organogenesis Medium (TOM), which consists of Murashige and Skoog salts (1962); B5 vitamins (Gamborg et

al., 1968); 2.5 mg l^{-1} BAP; 1 mg l^{-1} IAA; 87 mM sucrose; and 2 g l^{-1} GELRITE™ (Sigma, St. Louis, MO). Leaf discs co-cultivated with *A. tumefaciens* EHA105 lacking pKGA were used as a control. After two days of co-cultivation leaf discs were transferred to TOM-A (TOM containing 197 mM D-arabitol in place of sucrose). Leaf discs were transferred biweekly to TOM-A. After one month, a maximum of three regenerating shoots from each original leaf disc were placed on T⁻ rooting medium, which is the growth-regulator-free version of TOM.

Eighteen shoots were obtained from a total of six leaf discs co-cultivated with *A. tumefaciens* containing pKGA in one replicate and 13 shoots were obtained in a second replicate. Exhibit A shows the shoots from an original leaf disc. No shoots were obtained from control leaf tissue. Exhibit B shows a control leaf disc.

Rice transformation and selection

Embryogenic calli of rice, genotypes Tapei 309 and TN67, were cultured on N6 medium (Chu et al. 1975) supplemented with proline (500mg/L) and sucrose (87 mM). Plates containing 25-30 rice embryogenic calli were subjected to biolistics with pAS1305.2. Bombarded and control (non-bombarded) rice embryogenic calli were maintained for one week on N6 medium. Calli were then selected on modified N6 medium containing 164 mM arabitol and 15 mM sucrose and maintained in the dark. After 4 weeks, visibly growing calli were moved to selection medium containing 181 mM arabitol and 7 mM sucrose. After approximately 6-8 weeks of selection the calli were transferred onto regeneration medium.

Sixteen arabitol-selected cell clusters from a total of 170 embryogenic calli bombarded with pAS13052 were positive for the *atl*D gene by PCR. No growing cell clusters were obtained from control calli selected on arabitol.

2. The experiments discussed in item 1 above show that use of the positive selection techniques, taught in the subject application, provided a significant selective advantage for transformed cells. Transformation of the construct containing the arabitol dehydrogenase enzyme conferred the ability to grow and thrive in a selection medium containing arabitol as a nutrient source. This ability to thrive and grow was dramatic when compared to the control non-transformed cells, which showed a complete lack of

ability to thrive. Thus, in conducting the techniques and methods taught in the subject application, without question, one skilled in the art would be able to practice the subject invention without undue experimentation.

3. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information in belief are believed to be true; and further that these statements were made with the knowledge that willful false statements in the like so made are punishable by fine or imprisonment, or both, under §1001 of title 18 of the U.S.C. and that such willful false statements made jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Peter LaFayette, Ph.D.

10/20/03
Date